15.04.2003

PCT/GR03/00014

ΟΡΓΑΝΙΣΜΟΣ ΒΙΟΜΗΧΑΝΙΚΗΣ ΙΔΙΟΚΤΗΣΙΑΣ (ΟΒΙ)

ΠΙΣΤΟΠΟΙΗΤΙΚΟ

Βεβαιώνουμε ότι τα έγγραφα που συνοδεύουν το πιστοποιητικό αυτό, είναι ακριβή και πιστά αντίγραφα της κανονικής αίτησης για Δίπλωμα Ευρεσιτεχνίας, με αριθμό **20020100190**, που κατατέθηκε στον Οργανισμό Βιομηχανικής Ιδιοκτησίας στις **17/04/2002**, από το **Ελληνικό Ινστιτούτο Παστέρ**, Βας. Σοφίας 127, 11521, Αθήνα, από την **Association Francaise contre les Myopathies**, 13, Place de Rungis, 75013, Paris, France, από τον **κο Τζάρτο Σωκράτη**, Φορμίωνος 147, 16121, Καισαριανή και την **κα Μαμαλάκη Αυγή**, Τοσίτσα 15, 10683, Αθήνα.

Μαρούσι, 15/04/2003

Για τον Ο.Β.Ι.
Ο Γενικός Διευθυντής

Εμμανουήλ Σαμουηλίδης

ΠΑΝΤΑΝΑΣΣΗΣ 5, 151 25 ΠΑΡΑΔΕΙΣΟΣ ΑΜΑΡΟΥΣΙΟΥ - ΤΗΛ.: 61 83 506 - FAX: 68 19 231

ΟΡΓΑΝΙΣΜΟΣ ΒΙΟΜΗΧΑΝΙΚΗΣ ΙΔΙΟΚΤΗΣΙΑΣ

ΑΙΤΗΣΗ ΓΙΑ ΧΟΡΗΓΗΣΗ

ΔΙΠΛΩΜΑΤΟΣ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (ΔΕ)
Ή ΔΙΠΛΩΜΑΤΟΣ ΤΡΟΠΟΠΟΙΗΣΗΣ (ΔΤ)
Ή ΠΙΣΤΟΠΟΙΗΤΙΚΟΥ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (ΠΥΧ)

Αριθμός αίτησης:	20020100190
Ημερομηνία παραλαβής:	17/04/02
Ημερομηνία κατάθεσης:	17/04/02

Συμπληρώνεται από τον Ο.Β.Ι.

01

Με την αίτηση αυτή ζητείται:

02

- ☒ ΔΙΠΛΩΜΑ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (Δ.Ε.)
- ☐ ΔΙΠΛΩΜΑ ΤΡΟΠΟΠΟΙΗΣΗΣ (Δ.Τ.) ΣΤΟ Δ.Ε. με αριθμό:
- ☐ ΠΙΣΤΟΠΟΙΗΤΙΚΟ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (Π.Υ.Χ.)

03

Η αίτηση αυτή είναι τμηματική της αίτησης με αριθμό :

04

ΤΙΤΛΟΣ ΤΗΣ ΕΦΕΥΡΕΣΗΣ :

Παραγωγή ανασυνδυασμένων τμημάτων του μυϊκού υποδοχέα της ακετυλοχολίνης (AChr) και χρήση στην ex vivo ανοσοπροσρόφηση των αντισωμάτων έναντι του AChR από ασθενείς με βαριά μυασθένεια.

05

ΚΑΤΑΘΕΤΗΣ :

Επώνυμο ή επωνυμία:	Ελληνικό Ινστιτούτο Παστέρ (Ν.Π.Ι.Δ.) κατά ποσοστό 37%
Όνομα:	
Διεύθυνση/Έδρα:	Βασ. Σοφίας 127 1115 21 Αθήνα
Εθνικότητα:	Ελληνική
Τηλέφωνο:	010/647.8844 Φαξ: 647.8842 E-mail:

06

3 Αριθμός	ΕΠΙΠΛΕΟΝ ΚΑΤΑΘΕΤΕΣ ΣΕ ΠΡΟΣΘΕΤΟ ΦΥΛΛΟ ΧΑΡΤΙΟΥ

ΕΦΕΥΡΕΤΗΣ : 07

☐ Ο(ι) καταθέτης (ες) είναι ο(οι) μοναδικός(οί) εφευρέτης(ες).

☒ Έντυπο ορισμού του(ων) εφευρέτη(ών) επισυνάπτεται.

ΑΞΙΩΣΕΙΣ: 08

Αριθμός αξιώσεων: 30

ΔΗΛΩΣΗ ΠΡΟΤΕΡΑΙΟΤΗΤΑΣ 09

	Αριθμός	Ημερομηνία	Χώρα προέλευσης
1.			
2.			
3.			

ΑΛΛΕΣ

ΠΛΗΡΕΞΟΥΣΙΟΣ 10

Επώνυμο: ΚΟΡΙΑΤΟΠΟΥΛΟΥ
Όνομα: ΠΗΕΡΡΙΝΑ
Διεύθυνση: Ακαδημίας 16 10671 Αθήνα
Τηλέφωνο: 010/3614.857 Φαξ: 010/3615.041 E-mail:

ΑΝΤΙΚΛΗΤΟΣ 11

Επώνυμο: ΚΟΡΙΑΤΟΠΟΥΛΟΥ
Όνομα: ΠΗΕΡΡΙΝΑ
Διεύθυνση: Ακαδημίας 16 10671 Αθήνα
Τηλέφωνο: 010/3614.857 Φαξ: 010/3615.041 E-mail:

ΔΙΕΘΝΗΣ ΕΚΘΕΣΗ: 12

☐ Η εφεύρεση παρουσιάστηκε σε επίσημα αναγνωρισμένη έκθεση, σύμφωνα με το ν. 5562/1932, ΦΕΚ 221Α/32.

☐ Σχετική βεβαίωση επισυνάπτεται.

Τόπος: Αθήνα 13

Ημερομηνία: 17-04-2002

ΥΠΟΓΡΑΦΗ(ΕΣ) ΤΟΥ(ΩΝ) ΚΑΤΑΘΕΤΗ(ΩΝ) Ή ΤΟΥ(ΩΝ) ΠΛΗΡΕΞΟΥΣΙΟΥ(ΩΝ) :

ΠΗΕΡΡΙΝΑ Σ. ΚΟΡΙΑΤΟΠΟΥΛΟΥ
ΔΙΚΗΓΟΡΟΣ, Δ.Ν.
ΑΜ 13156
ΑΚΑΔΗΜΙΑΣ 16 ΑΘΗΝΑ 106 71
Τηλ. 3614857 - Fax. 3615041

ΠΑΡΑΚΑΛΟΥΜΕ Η ΑΙΤΗΣΗ ΝΑ ΕΙΝΑΙ ΔΑΚΤΥΛΟΓΡΑΦΗΜΕΝΗ ΚΑΘΩΣ ΚΑΙ ΤΟ ΟΝΟΜΑ ΚΑΤΩ ΑΠΟ ΤΗΝ ΥΠΟΓΡΑΦΗ. ΣΕ ΠΕΡΙΠΤΩΣΗ ΝΟΜΙΚΟΥ ΠΡΟΣΩΠΟΥ ΝΑ ΔΗΛΩΘΕΙ ΚΑΙ Η ΙΔΙΟΤΗΤΑ ΤΟΥ ΥΠΟΓΡΑΦΟΝΤΟΣ ΓΙΑ ΤΗΝ ΕΤΑΙΡΕΙΑ.

ΟΡΓΑΝΙΣΜΟΣ ΒΙΟΜΗΧΑΝΙΚΗΣ ΙΔΙΟΚΤΗΣΙΑΣ

ΕΝΤΥΠΟ ΓΙΑ ΕΠΙΠΛΕΟΝ ΚΑΤΑΘΕΤΕΣ

ΑΙΤΗΣΗΣ ΓΙΑ ΧΟΡΗΓΗΣΗ ΔΙΠΛΩΜΑΤΟΣ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (ΔΕ) Ή ΔΙΠΛΩΜΑΤΟΣ ΤΡΟΠΟΠΟΙΗΣΗΣ (ΔΤ) Ή ΠΙΣΤΟΠΟΙΗΤΙΚΟΥ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (ΠΥΧ)

Κ

Αριθμός αίτησης:	20020100190	01
Ημερομηνία παραλαβής:	17/04/02	
Ημερομηνία κατάθεσης:	17/04/02	

Συμπληρώνεται από τον Ο.Β.Ι.

ΚΑΤΑΘΕΤΗΣ :				05
Επώνυμο ή επωνυμία:	Association Française contre les Myopathies κατά ποσοστό 3%			
Όνομα:				
Διεύθυνση/Έδρα:	13, Place de Rungis 75013 Paris France			
Εθνικότητα:	Γαλλική			
Τηλέφωνο:	+1.4416.2700	Φαξ:	+1.4580.373	E-mail:

ΚΑΤΑΘΕΤΗΣ :				05
Επώνυμο ή επωνυμία:	ΤΖΑΡΤΟΣ κατά ποσοστό 55%			
Όνομα:	Σωκράτης			
Διεύθυνση/Έδρα:	Φορμίωνος 147 161 21 Καισαριανή			
Εθνικότητα:	Ελληνική			
Τηλέφωνο:	010/764.2241	Φαξ:		E-mail:

ΚΑΤΑΘΕΤΗΣ :				05
Επώνυμο ή επωνυμία:	ΜΑΜΑΛΑΚΗ κατά ποσοστό 5%			
Όνομα:	Αυγή			
Διεύθυνση/Έδρα:	Τοσίτσα 15 10683 Αθήνα			
Εθνικότητα:	Ελληνική			
Τηλέφωνο:	010.6478.835	Φαξ:		E-mail:

0 Αριθμός	ΕΠΙΠΛΕΟΝ ΚΑΤΑΘΕΤΕΣ ΣΕ ΠΡΟΣΘΕΤΟ ΦΥΛΛΟ ΧΑΡΤΙΟΥ	21

Κ

ΟΡΓΑΝΙΣΜΟΣ ΒΙΟΜΗΧΑΝΙΚΗΣ ΙΔΙΟΚΤΗΣΙΑΣ

ΟΡΙΣΜΟΣ ΤΟΥ ΕΦΕΥΡΕΤΗ

(ΣΥΜΠΛΗΡΩΝΕΤΑΙ ΣΤΗΝ ΠΕΡΙΠΤΩΣΗ ΠΟΥ Ο ΚΑΤΑΘΕΤΗΣ ΕΙΝΑΙ ΝΟΜΙΚΟ ΠΡΟΣΩΠΟ, Η Ο ΚΑΤΑΘΕΤΗΣ ΔΕΝ ΕΙΝΑΙ ΚΑΙ ΕΦΕΥΡΕΤΗΣ, Η Ο ΜΟΝΟΣ ΕΦΕΥΡΕΤΗΣ)

Τ.Β.Ο. και σια ΜΕΣΑΙΩΝΙΤΗΣ

Αριθμός αίτησης:	20020100190	01
Ημερομηνία παραλαβής:	17-04-02	
Ημερομηνία κατάθεσης:	17-04-02	

☒	ΔΙΠΛΩΜΑ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (Δ.Ε.)	02
☐	ΔΙΠΛΩΜΑ ΤΡΟΠΟΠΟΙΗΣΗΣ (Δ.Τ.) ΣΤΟ Δ.Ε. με αριθμό:	
☐	ΠΙΣΤΟΠΟΙΗΤΙΚΟ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (Π.Υ.Χ.)	

Δηλώνω(νουμε) ως εφευρέτης(ες) στην παραπάνω αίτηση για χορήγηση Ελληνικού τίτλου προστασίας του(τους) :

ΕΦΕΥΡΕΤΗΣ 22

Επώνυμο: ΤΖΑΡΤΟΣ

Όνομα: ΣΩΚΡΑΤΗΣ

Διεύθυνση: Φορμίωνος 147 16121 Καισαριανή Αττικής

Ο καταθέτης απέκτησε το δικαίωμα κατάθεσης Ελληνικού τίτλου προστασίας :

- ☒ Λόγω σύμβασης μεταβίβασης δικαιωμάτων από : 15-04-2002 (ημερομηνία)
- ☐ Λόγω κληρονομικής διαδοχής
- ☒ Λόγω συμβατικής σχέσης εργοδότη – εργαζόμενου (υπηρεσιακής ☐ ή εξαρτημένης ☒)
- ☐ Με βάση το καταστατικό της εταιρίας
- ☐

3 Αριθμός	ΕΠΙΠΛΕΟΝ ΕΦΕΥΡΕΤΕΣ ΣΕ ΠΡΟΣΘΕΤΟ ΦΥΛΛΟ ΧΑΡΤΙΟΥ	23

Τόπος: Αθήνα

Ημερομηνία: 25-07-2002

ΥΠΟΓΡΑΦΗ(ΕΣ) ΤΟΥ(ΩΝ) ΚΑΤΑΘΕΤΗ(ΩΝ) ή ΤΟΥ(ΩΝ) ΠΛΗΡΕΞΟΥΣΙΟΥ(ΩΝ) : 24

ΠΗΝΕΛΟΠΗ Σ. ΚΟΡΙΑΤΟΠΟΥΛΟΥ
ΔΙΚΗΓΟΡΟΣ, Δ.Ν.
ΑΜ 13156
ΑΚΑΔΗΜΙΑΣ 16 ΑΘΗΝΑ 106 71
Τηλ. 3614357 - Fax. 3615041

ΠΑΡΑΚΑΛΟΥΜΕ Η ΑΙΤΗΣΗ ΝΑ ΕΙΝΑΙ ΔΑΚΤΥΛΟΓΡΑΦΗΜΕΝΗ ΚΑΘΩΣ ΚΑΙ ΤΟ ΟΝΟΜΑ ΚΑΤΩ ΑΠΟ ΤΗΝ ΥΠΟΓΡΑΦΗ. ΣΕ ΠΕΡΙΠΤΩΣΗ ΝΟΜΙΚΟΥ ΠΡΟΣΩΠΟΥ ΝΑ ΔΗΛΩΘΕΙ ΚΑΙ Η ΙΔΙΟΤΗΤΑ ΤΟΥ ΥΠΟΓΡΑΦΟΝΤΟΣ ΓΙΑ ΤΗΝ ΕΤΑΙΡΕΙΑ.

ΟΡΓΑΝΙΣΜΟΣ
ΒΙΟΜΗΧΑΝΙΚΗΣ
ΙΔΙΟΚΤΗΣΙΑΣ

ΟΡΙΣΜΟΣ ΤΟΥ ΕΦΕΥΡΕΤΗ

(ΣΥΜΠΛΗΡΩΝΕΤΑΙ ΣΤΗΝ ΠΕΡΙΠΤΩΣΗ ΠΟΥ Ο ΚΑΤΑΘΕΤΗΣ ΕΙΝΑΙ ΝΟΜΙΚΟ ΠΡΟΣΩΠΟ,
Η Ο ΚΑΤΑΘΕΤΗΣ ΔΕΝ ΕΙΝΑΙ ΚΑΙ ΕΦΕΥΡΕΤΗΣ, Η Ο ΜΟΝΟΣ ΕΦΕΥΡΕΤΗΣ)

Από τον Ο.Β.Ι.

Αριθμός αίτησης:	20020100190	01
Ημερομηνία παραλαβής:	17-04-02	
Ημερομηνία κατάθεσης:	17-04-02	

☒	ΔΙΠΛΩΜΑ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (Δ.Ε.)	02
☐	ΔΙΠΛΩΜΑ ΤΡΟΠΟΠΟΙΗΣΗΣ (Δ.Τ.) ΣΤΟ Δ.Ε. με αριθμό:	
☐	ΠΙΣΤΟΠΟΙΗΤΙΚΟ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (Π.Υ.Χ.)	

Δηλώνω(νουμε) ως εφευρέτης(ες) στην παραπάνω αίτηση για χορήγηση Ελληνικού τίτλου προστασίας του(τους) :

ΕΦΕΥΡΕΤΗΣ 22

Επώνυμο: ΜΑΜΑΛΑΚΗ

Όνομα: ΑΥΓΗ

Διεύθυνση: Τοσίτσα 15 10683 Αθήνα

Ο καταθέτης απέκτησε το δικαίωμα κατάθεσης Ελληνικού τίτλου προστασίας :

- ☒ Λόγω σύμβασης μεταβίβασης δικαιωμάτων από : 15-04-2002 (ημερομηνία)
- ☐ Λόγω κληρονομικής διαδοχής
- ☒ Λόγω συμβατικής σχέσης εργοδότη – εργαζόμενου (υπηρεσιακής ☒ ή εξαρτημένης ☐)
- ☐ Με βάση το καταστατικό της εταιρίας
- ☐

2 Αριθμός	ΕΠΙΠΛΕΟΝ ΕΦΕΥΡΕΤΕΣ ΣΕ ΠΡΟΣΘΕΤΟ ΦΥΛΛΟ ΧΑΡΤΙΟΥ	23

Τόπος: Αθήνα

Ημερομηνία: 25-07-2002

ΥΠΟΓΡΑΦΗ(ΕΣ) ΤΟΥ(ΩΝ) ΚΑΤΑΘΕΤΗ(ΩΝ) ή ΤΟΥ(ΩΝ) ΠΛΗΡΕΞΟΥΣΙΟΥ(ΩΝ) : 24

ΠΙΕΡΡΙΝΑ Σ. ΚΟΡΙΑΤΟΠΟΥΛΟΥ
ΔΙΚΗΓΟΡΟΣ, Δ.Ν.
ΑΜ 13156
ΑΚΑΔΗΜΙΑΣ 16 ΑΘΗΝΑ 106 71
Τηλ. 3614857 - Fax. 3615041

ΠΑΡΑΚΑΛΟΥΜΕ Η ΑΙΤΗΣΗ ΝΑ ΕΙΝΑΙ ΔΑΚΤΥΛΟΓΡΑΦΗΜΕΝΗ ΚΑΘΩΣ ΚΑΙ ΤΟ ΟΝΟΜΑ ΚΑΤΩ ΑΠΟ ΤΗΝ ΥΠΟΓΡΑΦΗ. ΣΕ ΠΕΡΙΠΤΩΣΗ ΝΟΜΙΚΟΥ ΠΡΟΣΩΠΟΥ ΝΑ ΔΗΛΩΘΕΙ ΚΑΙ Η ΙΔΙΟΤΗΤΑ ΤΟΥ ΥΠΟΓΡΑΦΟΝΤΟΣ ΓΙΑ ΤΗΝ ΕΤΑΙΡΕΙΑ.

ΟΡΓΑΝΙΣΜΟΣ ΒΙΟΜΗΧΑΝΙΚΗΣ ΙΔΙΟΚΤΗΣΙΑΣ

ΟΡΙΣΜΟΣ ΤΟΥ ΕΦΕΥΡΕΤΗ

Ε

(ΣΥΜΠΛΗΡΩΝΕΤΑΙ ΣΤΗΝ ΠΕΡΙΠΤΩΣΗ ΠΟΥ Ο ΚΑΤΑΘΕΤΗΣ ΕΙΝΑΙ ΝΟΜΙΚΟ ΠΡΟΣΩΠΟ, Η Ο ΚΑΤΑΘΕΤΗΣ ΔΕΝ ΕΙΝΑΙ ΚΑΙ ΕΦΕΥΡΕΤΗΣ, Η Ο ΜΟΝΟΣ ΕΦΕΥΡΕΤΗΣ)

www.obi.gr Ο.Β.Ι.

Αριθμός αίτησης:	20020100190	01
Ημερομηνία παραλαβής:	17-04-02	
Ημερομηνία κατάθεσης:	17-04-02	

☒	ΔΙΠΛΩΜΑ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (Δ.Ε.)	02
☐	ΔΙΠΛΩΜΑ ΤΡΟΠΟΠΟΙΗΣΗΣ (Δ.Τ.) ΣΤΟ Δ.Ε. με αριθμό:	
☐	ΠΙΣΤΟΠΟΙΗΤΙΚΟ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (Π.Υ.Χ.)	

Δηλώνω(νουμε) ως εφευρέτης(ες) στην παραπάνω αίτηση για χορήγηση Ελληνικού τίτλου προστασίας του(τους) :

ΕΦΕΥΡΕΤΗΣ 22

Επώνυμο: ΨΑΡΙΔΗ - ΛΙΝΑΡΔΑΚΗ

Όνομα: ΛΟΥΚΙΑ

Διεύθυνση: Π. Λινού 14 17563 Π. Φάληρο

Ο καταθέτης απέκτησε το δικαίωμα κατάθεσης Ελληνικού τίτλου προστασίας :

- ☒ Λόγω σύμβασης μεταβίβασης δικαιωμάτων από : 15-04-2002 (ημερομηνία)
- ☐ Λόγω κληρονομικής διαδοχής
- ☒ Λόγω συμβατικής σχέσης εργοδότη – εργαζόμενου (υπηρεσιακής ☒ ή εξαρτημένης ☐)
- ☐ Με βάση το καταστατικό της εταιρίας
- ☐

1 Αριθμός	ΕΠΙΠΛΕΟΝ ΕΦΕΥΡΕΤΕΣ ΣΕ ΠΡΟΣΘΕΤΟ ΦΥΛΛΟ ΧΑΡΤΙΟΥ	23

Τόπος: Αθήνα

Ημερομηνία: 25-07-2002

ΥΠΟΓΡΑΦΗ(ΕΣ) ΤΟΥ(ΩΝ) ΚΑΤΑΘΕΤΗ(ΩΝ) ή ΤΟΥ(ΩΝ) ΠΛΗΡΕΞΟΥΣΙΟΥ(ΩΝ) : 24

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ΟΡΙΣΜΟΣ ΤΟΥ ΕΦΕΥΡΕΤΗ

(ΣΥΜΠΛΗΡΩΝΕΤΑΙ ΣΤΗΝ ΠΕΡΙΠΤΩΣΗ ΠΟΥ Ο ΚΑΤΑΘΕΤΗΣ ΕΙΝΑΙ ΝΟΜΙΚΟ ΠΡΟΣΩΠΟ,
Η Ο ΚΑΤΑΘΕΤΗΣ ΔΕΝ ΕΙΝΑΙ ΚΑΙ ΕΦΕΥΡΕΤΗΣ, Η Ο ΜΟΝΟΣ ΕΦΕΥΡΕΤΗΣ)

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☒	ΔΙΠΛΩΜΑ ΕΥΡΕΣΙΤΕΧΝΙΑΣ (Δ.Ε.)	02
☐	ΔΙΠΛΩΜΑ ΤΡΟΠΟΠΟΙΗΣΗΣ (Δ.Τ.) ΣΤΟ Δ.Ε. με αριθμό:	
☐	ΠΙΣΤΟΠΟΙΗΤΙΚΟ ΥΠΟΔΕΙΓΜΑΤΟΣ ΧΡΗΣΙΜΟΤΗΤΑΣ (Π.Υ.Χ.)	

Δηλώνω(νουμε) ως εφευρέτης(ες) στην παραπάνω αίτηση για χορήγηση Ελληνικού τίτλου προστασίας του(τους) :

22

ΕΦΕΥΡΕΤΗΣ

Επώνυμο: ΚΩΣΤΕΛΙΔΟΥ

Όνομα: ΚΑΛΛΙΟΠΗ

Διεύθυνση: Απω Ανατολής 32 Α 16562 Γλυφάδα Αττικής

Ο καταθέτης απέκτησε το δικαίωμα κατάθεσης Ελληνικού τίτλου προστασίας :

- ☒ Λόγω σύμβασης μεταβίβασης δικαιωμάτων από : 15-04-2002 (ημερομηνία)
- ☐ Λόγω κληρονομικής διαδοχής
- ☒ Λόγω συμβατικής σχέσης εργοδότη – εργαζόμενου (υπηρεσιακής ☒ ή εξαρτημένης ☐)
- ☐ Με βάση το καταστατικό της εταιρίας
- ☐

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Αριθμός	ΕΠΙΠΛΕΟΝ ΕΦΕΥΡΕΤΕΣ ΣΕ ΠΡΟΣΘΕΤΟ ΦΥΛΛΟ ΧΑΡΤΙΟΥ

24

Τόπος: Αθήνα

Ημερομηνία: 25-07-2002

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20020100190

Title:

Production of recombinant fragments of muscle acetylcholine receptor and their use for *ex vivo* immunoadsorption of anti-ACh receptor antibodies from myasthenic patients

DESCRIPTION

Field of the invention

This invention relates to the field of extacorporeal blood processing, and more specifically to the antigen-specific elimination of autoantibodies for the treatment of autoimmune diseases.

Background of the invention

Myasthenia gravis (**MG**) is a well characterized neurological autoimmune disease. It affects at least one out of 8.000 people. In MG, the characteristic weakness and fatiguability of the voluntary muscles result from antibody-mediated loss of the acetylcholine receptors (AChR) at the neuromuscular junction (Karlin et al, 1995, *Neuron* 15, 1231-1244). If swallowing and breathing muscles are involved, it can still be life-threatening. The pathogenic role of serum antibodies is clearly demonstrated by the dramatic clinical improvement that follows plasma exchange and immunosuppressive treatments. The AChR is a transmembrane glycoprotein consisting of five homologous subunits with the stoichiometry of $alpha_2beta,gamma,delta$ or $alpha_2beta,epsilon,delta$ (Devillers-Thiery et al, 1993, *J. Membr. Biol.* 3, 133-191; Karlin, 1993, *Curr. Opin. Neurobiol.* 3, 299-309). Effector mechanisms responsible for antibody-dependent AChR loss are: (a) cross-linking of AChRs by bivalent antibodies; (b) activation of complement; and less importantly (c) direct interference with the ion channel (Willcox, 1993, *Curr. Opin. Immunol.* 5: 910-917 ; Tzartos et al, 1998, *Immunol. Rev.* 163, 89-120). The mechanism that triggers the autoimmune response to the AChR is not known.

Current treatments of MG include anticholinesterase and immunosuppressive agents, thymectomy, plasmapheresis and intravenous human Ig (Massey, 1997, *Neurology*, 48, S46-S51). Although these therapies are often successful, they are non-specific and can be associated with severe side effects. Ideally, therapy should be antigen-specific and aimed at preventing the induction of or eliminating the specific antibodies.

Plasmapheresis is an efficient short-term treatment for MG, and clinical improvement correlates well with the reduction in circulating anti-AChR antibodies(Yeh et al 1999, *Acta Neurol. Scand.*, 100, 305-309). However, plasmapheresis is very expensive, because of the need to replace serum proteins, and removes non-pathogenic (protective) antibodies and other important molecules. An affinity column made of AChR or appropriate AChR fragments that bind selectively the anti-AChR antibodies would overcome many of these

problems. Since native AChR is available only in very small quantities, specific immunoadsorption can only be achieved by the achieved by the use of columns with recombinant AChR fragments. Because binding of the majority of the anti-AChR antibodies of MG patients is highly conformation dependent, denatured recombinant AChR fragments, like those usually obtained by E. coli expression systems or synthetic peptides, are unable to bind significant fractions of the anti-AChR MG antibodies. Therefore, AChR fragments of near-native conformation are needed, and such may be obtained by eucaryotic expression systems. Although most eucaryotic expression systems produce inappropriately low concentrations there are a few systems which allow considerable production of recombinant proteins. These include the yeast *Pichia pastoris* and the Semliki Forest Virus (SFV) expression systems.

Recombinant subunits, or their fragments, of non-human muscle AChRs have been produced in mammalian cells but in small amounts. These proteins bind some conformation dependent acetylcholine receptor monoclonal antibodies but their ability to bind MG sera was not tested. Non-human AChR subunits have been also produced in Sf9 cells by the baculovirus expression system and in the yeast Saccharomyces expression system; no binding of MG antibodies was determined. In any case, non-human AChRs generally are not able for binding major fractions of the MG antibodies.

Immunoadsorbent columns with resin-linked tryptophan (TR 350$^{@}$), phenylalanine or protein A (Immunosorba.RTM, Excorim.RTM, Lund, Sweden) have been used in MG and in other autoimmune diseases. These columns immunoadsorb large fractions of the plasma IgG together with similar or larger fractions of anti-AChR antibodies. Yet this procedure is non-specific and eliminates useful immunoglobulins from the plasma.

Despite the general observation that MG antibodies require folded human AChR domains in order to bind satisfactorily, a column has been constructed (MG50$^{@}$) on which the Torpedo AChR alpha subunit segment 183-200 (which contains part of the ligand binding site of the AChR) has been immobilized. Such columns have been used as immunoadsorbents for MG treatment (Takamori et al, 1996, *Transfus Sci.* 17, 445-53). Yet the immunoadsorption efficiency of such columns is questionable.

Our approach aims at the production of recombinant AChR fragments capable of binding the majority of the MG antibodies, for use as highly specific immunoadsorbents for the *ex vivo* selective elimination of the patients' anti-AChR antibodies. Since the great majority of the anti-AChR antibodies of MG patients bind extracellularly (Tzartos et al, 1980, *Proc. Natl. Acad. Sci. USA* 77, 755-759; Tzartos et al 1982, *Proc. Natl. Acad. Sci. USA* 79, 188-192), and since only these antibodies can be pathogenic, we are expressing only the extracellular parts of the human AChR subunits as being water - soluble proteins and therefore more conveniently

used. However, expression of larger fragments or intact subunits containing the extracellular domains, or smaller fragments containing large parts of the extracellular domains of the AChR subunits, will apparently behave similarly and are covered by this patent application.

Summary of the invention

The N-terminal extracellular domains (for example amino acids 1-210, 1-211) of the alpha, beta, gamma, delta and epsilon subunits of the human muscle AChR, are expressed in highly efficient eucariotic systems including the yeast *Pichia pastoris.* The polypeptides are water-soluble, glycosylated, in the form of monomers. Alpha1-210 binds ^{125}I-alpha-bungarotoxin with high affinity. Moreover, this binding is efficiently blocked by unlabeled d-tubocurarine and gallamine. Several conformation-dependent anti-AChR monoclonal antibodies are able to bind to this proteins as do antibodies from a large proportion of MG patients. It should be noted that Alpha1-210 is the first recombinant fragment of human AChR alpha subunit expressed in eucariotic systems that combines near-native conformation together with relatively high expression yield. The purified protein is subsequently immobilized on insoluble carriers (for example Sepharose-CNBr) and is used to immunoadsorb anti-AChR antibodies from MG sera. In the immunoadsorbent column that we propose the alpha subunit fragment eliminates more than 60% (60-94%) of the anti-AChR antibodies in 20% of the sera, whereas from another 30% of the sera it eliminates 20-60% of their anti-AChR antibodies. The non-alpha proteins might also eliminate significant fractions of MG antibodies. Their combined use should be able to eliminate the great majority of the anti-AChR antibodies from most MG patients and to be used in ex vivo antibody-apheresis for MG treatment.

Brief description of the drawings

Figure. 1 illustrates the constructs used (A) and the glycosylation (B) deglycosylation (C) of Alpha1-210. (A) The human alpha subunit extracellular domain, residues 1-210, was led by a signal peptide α-factor under the transcriptional control of the AOX promoter, which is cleaved by host enzymes (▼represents the cleavage site), while, at the C-terminal, it was fused to a c-myc epitope and a 6-residue polyhistidine tag. (B) Purification of Alpha1-210 using NiNTA metal affinity chromatography: Purified Alpha1-210 was analysed by 12 % SDS PAGE (lane 1) and the proteins stained with Coomassie Brilliant Blue; the positions of molecular weight markers are indicated (lane 2). (C) Deglycosylation of Alpha1-210 by peptide N-glycosidase F. Purified Alpha1-210 was incubated at 37° C for 6 hours in the absence (lane 1) or presence (lane 2) of N-glycosidase F, then the mixture was analysed by 12 % SDS PAGE followed by Western

blotting using mAb 198. The arrows indicate the band corresponding to the glycosylated (upper) and deglycosylated (lower) forms of Alpha1-210.

Figure. 2 illustrates the binding of alpha-BTX to Alpha1-210. (A) Scatchard plot analysis of ^{125}I-alpha -BTX binding to Alpha1-210 fragment. Culture supernatant containing Alpha1-210 (40 µl) was incubated with various concentrations of ^{125}I- alpha -BTX in 10 µl of PBS–0.2 % BSA, then, after 3 hours incubation at 4° C, the samples were filtered through Whatman filters as described above, and the bound radioactivity measured on a γ counter. Samples were processed in duplicates. The estimated Kd in the representative experiment shown is 3,3 nM while the average Kd of a series of experiments was 5.1 ± 2.4 nM. (B) The specific binding of the ^{125}I-alpha-BTX to Alpha1-210 fragment was studied in competition experiments involving the simultaneous addition of increasing concentrations of unlabeled toxin. Samples were processed in duplicates. Binding of labeled alpha-BTX in the absence of unlabeled alpha-BTX was taken as the 100 % level. (C) Effect of deglycosylation on ^{125}I-alpha-BTX binding to Alpha1-210. *In vitro* deglycosylation was performed using N-glycosidase F treatment. Aliquots at various reaction time-points were assayed for ^{125}I- alpha-BTX binding in filter assay experiments. Samples were processed in duplicates (▨ N-glycosidase F treatment; ▩ control treatment).

Figure. 3 illustrates the binding of cholinergic ligands to Alpha1-210: Different concentrations of unlabeled nicotine (–·◆·–), carbamylcholine (—▲—), d-tubocurarine (—▫—), or gallamine (–●–·) were added at the same time as ^{125}I-alpha-BTX to Alpha1-210 in competition binding studies. Gallamine and d-tubocurarine inhibited ^{125}I-alpha-BTX binding at concentrations higher than 7 mM (Ki ~ 7.5 mM), while neither carbamylcholine nor nicotine competed with ^{125}I-alpha-BTX. NaCl (···▽····) was used to detect any non-specific effect of increasing ion strength. Samples were processed in duplicates. Binding of labeled alpha-BTX in the absence of any unlabeled ligand was taken as the 100 % level. The percentage binding is plotted against the ligand concentration.

Figure. 4 illustrates FPLC analysis of Alpha1-210. FPLC-purified Alpha1-210 has a molecular weight of ~ 34 kD. Gel filtration fractions (Superose-12 column, PBS buffer, pH 7.4, flow rate 0.5 ml/min) were assayed for ^{125}I-alpha-BTX binding. The arrows indicate fractions corresponding to polypeptides of known molecular weight.

Figure. 5 illustrates the binding of conformation dependent and partially conformation-dependent mAbs to Alpha1-210 fragment. Three groups of mAbs were used in RIA assays: a. anti-MIR mAbs, the binding of which is partially conformation-dependent (mAbs 198, 202, 195) (▨), b. anti-MIR mAbs that bind only to native receptor (mAbs 192, 190, 35) (▩), and c. mAb 64, an anti-α subunit mAb that does not bind to the MIR (□) and the binding of which is

conformation-dependent. mAb 25 was used as a negative control (■). Samples were processed in duplicates.

Figure. 6 illustrates the binding of anti-alpha autoantibodies from a high proportion of MG sera to recombinant Alpha1-210. 50 MG-positive sera were assayed for binding to Alpha1-210 and hybrid AChR (HαTβγδ containing human alpha subunit and *Torpedo* beta, gamma and delta subunits labeled with ^{125}I-alpha-BTX. MG sera binding to ^{125}I-alpha-BTX-Alpha1-210 was plotted versus binding to ^{125}I-alpha-BTX-HαTβγδ in a logarithmic scale. A regression line was fitted to these data with r = 0.74. Samples were processed in duplicates.

Figure. 7 illustrates the immunoadsorption of anti-AChR antibodies from MG sera using immobilized Alpha1-210. 64 MG positive sera were assayed for binding to human AChR after incubation with Alpha1-210 immobilized to CNBr-Sepharose or with BSA immobilized to the same insoluble matrix. 2.5µl MG patient sera were incubated with 0.2µg immobilized Alpha1-210 mixed with BSA or the same amount of BSA at 4°C for 2-3h. 0.9 µl of these treated MG sera were subsequently used in a typical RIA MG diagnostic test. The percentage of immunoadsorption was estimated as the ratio between the reduction in immunoprecipitated radiolabeled human AChR after incubation with Alpha1-210 and the immunoprecipitated radiolabeled human AChR without incubation with Alpha1-210 (incubation with just BSA). Samples were processed in duplicates.

Detailed description of the invention

The N-terminal extracellular domains (or larger pieces containing the said domains, or smaller pieces within the said domains) of the subunits of the human muscle AChR, are expressed in eucaryotic systems. Of these, the alpha subunit extracellular domain bears the binding sites for cholinergic ligands and the main immunogenic region (MIR), the major target for anti-AChR antibodies in MG patients. As examples, we used the expression systems of the yeast *Pichia pastoris* and of the Semliki Forest Virus (SFV).

The recombinant alpha subunit domain is expressed in a soluble form, and purified from the culture supernatant by affinity chromatography (Fig 1B) or by ion exchange or gel filtration chromatography (Fig 4). The molecular weight of the product is estimated as higher than that predicted from the amino acid sequence, this difference being due to glycosylation of the molecule at residue 141, since enzymatic deglycosylation using peptide N-glycosidase F results in a reduction in the apparent molecular weight (Fig. 1C). These results show that, like the native receptor alpha subunit, Alpha1-210 is glycosylated (Fujita et al 1986, *Science* 231, 1284-1287).

An indication of the quality of conformation of the human alpha subunit fragment is obtained from studying binding of ^{125}I-alpha-

bungarotoxin to the protein. Although E. coli expressed Torpedo extracellular alpha subunit fragment, especially after refolding, binds well ^{125}I-alpha-bungarotoxin, this does not happen with similarly expressed human alpha subunit fragments, even after refolding procedures (Tsouloufis et al, 2000, *Int. Immunol.* 12, 1255-1265). To study the binding of ^{125}I-alpha-bungarotoxin to Alpha1-210 as an indication of correct protein folding, we use a filter assay experiment (Fig 2A). A high affinity for alpha-bungarotoxin is obtained. However, when the protein is deglycosylated by an appropriate enzyme, ^{125}I-alpha-bungarotoxin ability is practically totally lost (Fig 2C).

Although alpha subunit alone does not contain the whole ligand binding site, some small ligands may be able to displace binding of ^{125}I-alpha-bungarotoxin (Fig. 3). This is the case with two small ligands described in Example 1.

An important parameter of the quality of conformation of the protein is its water-solubility. As described in Example 1 and Fig 4, gel filtration analysis shows that the purified protein is water-soluble and migrates as a monomer.

Before evaluating the binding of MG sera to Alpha1-210, the binding of several conformation-dependent anti-MIR and non-MIR monoclonal antibodies (mAbs) should be preferably tested. As it is shown in Fig. 5 and described in Example 1, all tested mAbs (against the extracellular part of the alpha subunit) are able to bind to the protein, further suggesting that the molecule assumes the correct conformation.

Other expression systems may be also used, as for example described in the Example 4. Similarly, the extracellular parts of the non-alpha AChR subunits may be expressed as for example is described in the Example 2 for the subunits beta, gamma, delta and epsilon expressed in the *Pichia pastoris* system.

Since the antibody- and ligand-binding experiments indicate that the recombinant proteins assume the correct conformation, one can study the binding of anti-AChR autoantibodies from MG sera to the recombinant proteins, since these antibodies are, in general, highly conformation-dependent (Tzartos et 1998). Example 1 describes the screening of 70 human sera (50 MG and 20 healthy controls). Their binding to the recombinant protein is compared to that on a solubilized hybrid AChR, containing human alpha subunit and *Torpedo* beta, gamma and delta subunits; since MG sera essentially do not bind to *Torpedo* AChR (Loutrari et al 1997, *Clin. Exp. Immunol.* 109, 538-546), their binding to the hybrid molecule represents binding to the alpha subunit in the hybrid AChR molecule. By this procedure we are able to compare antibody binding to the alpha subunit inserted in the intact AChR molecule and to the isolated recombinant alpha subunit fragment. Most sera are found to recognize the recombinant protein and this binding seems proportional to their binding to the alpha subunit on the human/Torpedo AChR hybrid, suggesting that a major fraction of the anti-alpha autoantibodies in MG patients bind to recombinant Alpha1-210.

Then the purified polypeptide is immobilized on an insoluble matrix (for example CNBr-Sepharose) and is used for the immunoadsorption of anti-AChR antibodies from MG sera and plasma. Interestingly, in the Example 3 and Fig. 7 is shown that the immobilized alpha domain is able to immunoadsorb more than 60% of the anti-AChR antibodies in several MG sera. Since plasmapheresis has beneficial effects with even partial withdrawal of the anti-AChR antibodies and partial reduction of the patient's titer, and since it is known that for a single patient anti-AChR antibody titers correlate well with MG symptoms, it is expected that elimination of even 60% of the anti-AChR antibodies will be highly beneficial to the patients. Moreover, the combined use with the extracellular parts of the other (non-alpha) subunits could increase the efficiency of the immunoadsorbents.

For more stable fixation of the polypeptide on the column and for avoidance of release of any possible fragments of it, the polypeptide-Sepharose complex is treated by a crosslinking reagent.

Expression of larger fragments or intact subunits containing the extracellular domains, or smaller fragments containing large parts of the extracellular domains of the AChR subunits, will apparently behave similarly and are obviously covered by this patent application. Below are provided some examples to prove the feasibility of the approach.

Example 1. Expression of extracellular domain of human AChR alpha subunit in yeast *Pichia pastoris*, its purification and characterization.

The N-terminal extracellular domain of the human muscle nicotinic AChR alpha subunit (Alpha 1-210) was expressed in yeast *Pichia pastoris*. The yeast expression system allows post-translational modifications including glycosylation, thus resulting in correct protein folding, while the ease of manipulation, short doubling time, and high yield of protein expression are similar to those using bacteria. We used the methylotropic yeast strain, *Pichia pastoris*, as it has a strong inducible promoter and it is less prone to hyperglycosylation than *Saccharomyces cerevisiae.*

Alpha 1-210 was enzymatically amplified by PCR using a full-length α1 cDNA clone. The upstream (5'-GCTGGCCTCGTCGAATT-CTCCGAACATG-3') and downstream (5'-GATGAAGTAGAGGTCTAGACGCTGCATGACG-3') primers were constructed to contain EcoR*I* and Xba*I* restriction sites (underlined). Using the appropriate restriction endonucleases, the purified cDNA fragment was subcloned into the expression vector pPICZαA (Invitrogen San Diego, CA) so that the recombinant fragment was led by a signal peptide α-factor under the transcriptional control of the AOX promoter, which is induced by methanol, while at the C terminal end it was fused to a sequence encoding the c-myc epitope and polyhistidine (6xHis) tag (Fig. 1A). The resulting construct was linearized using Pme*I* and transformed into the *Pichia pastoris* host

strain GS115 by electroporation (BioRad GenePulser). The transformed cells were plated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, and 1M sorbitol) plus zeocin (100 µg/ml) and incubated at 30°C for 3 days.

Single colonies of the transformants were initially inoculated into 4 ml of BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4 X 10^{-5}% biotin, and 1% glycerol). After 16-20 h at 30°C, the cells were resuspended in 4 ml of BMMY (identical to BMGY, except that the glycerol was replaced by 0.5% methanol) to induce expression. After induction for 3 days with daily addition of methanol (0.5% v/v), the culture supernatants were tested for expression of Alpha1-210 by dot-blot analysis using the anti-myc 9E.10 mAb (ATCC). The clone with the highest protein yield was used for large-scale protein expression.

The culture supernatant was concentrated using a Minitan Ultrafiltration System (Millipore) and dialyzed against PBS, pH 8, then Alpha1-210 was purified using NiNTA metal affinity chromatography (Qiagen) according to the manufacturer's protocol, the recombinant protein being eluted under native conditions using increasing imidazole concentrations (40, 70, and 100 mM). The eluates were analyzed by 12% SDS-PAGE followed by Coomassie Brilliant Blue staining (Fig. 1B) or Western blot analysis using anti-alpha subunit mAb, mAb198 or the anti-myc 9E.10 mAb. The protein concentration was determined using the Bradford method (BioRad) and the yield of purified Alpha1-210 was estimated to 0.2-0.3 mg/l. The molecular weight of the product was estimated as 34 kD, higher than that predicted from the amino acid sequence (27 kD); as reported previously, this difference was shown to be due to glycosylation of the molecule at residue 141, since enzymatic deglycosylation using peptide N-glycosidase F (PNGase F, New England Biolabs) resulted in a reduction in the apparent molecular weight to about 27 kD (Fig. 1C). These results show that, like the native receptor alpha subunit, Alpha1-210 is glycosylated.

High-affinity binding of ^{125}I-alpha-BTX to purified Alpha1-210.

To study the binding of alpha-BTX to Alpha1-210 as an indication of correct protein folding, we tested both crude culture supernatants and the purified protein in filter assay experiments. Fifty nanograms of purified Alpha1-210 or 20-40 ml of culture supernatant was incubated at 4°C for 3 h with various concentrations of ^{125}I-alpha-BTX (specific activity 800,000 cpm/pmole) in a final volume of 50 ml of PBS buffer, pH 7.5, 0.2% BSA. The samples were then diluted with 1 ml of 0.5% Triton X-100 in 20 mM Tris buffer, pH 7.5 (Triton buffer) and immediately filtered through two Whatman DE81 filters prewashed with Triton buffer. The filters were then washed twice with 1 ml of Triton buffer and the bound radioactivity measured on a γ-counter. Samples without Alpha1-210 were used to measure non-specific binding. Specific binding of alpha-BTX was also studied in competition experiments by addition of various amounts of unlabeled alpha-BTX to the sample.

As shown in Fig. 2A, Alpha1-210 bound alpha-BTX with a Kd, estimated by Scatchard analysis, of 5.1 ± 2.4 nM, which is only one order of magnitude lower than that of the native human AChR.

The binding of ^{125}I-alpha-BTX to Alpha1-210 was shown to be specific using various concentrations of unlabeled alpha-BTX in competition assays (Fig. 2B). Unlabeled toxin inhibited the binding of labeled alpha-BTX even at low concentrations. Moreover, the results were consistent with the Kd estimated from the Scatchard plot, since 50% inhibition of ^{125}I-alpha-BTX binding was seen using unlabeled toxin at a concentration of 5 nM. Interestingly, alpha-BTX binding was markedly impaired by *in vitro* deglycosylation of Alpha1-210 (Fig. 2C)

Ligand binding studies.

To further confirm the native-like conformation of the Alpha1-210 fragment, four cholinergic ligands were tested for inhibition of alpha-BTX binding to Alpha1-210. Various concentrations (0.1-20 mM) of carbamylcholine, nicotine, gallamine, or d-tubocurarine were added together with ^{125}I-alpha-BTX (5 nM) to Alpha1-210 (50 ng) in a final volume of 50 µl, and incubating the mixture at 4°C for 3 h The same concentrations of NaCl were used to detect any non-specific effect of an increase in ionic strength on alpha-BTX binding. Bound radioactivity was then measured using Whatman DE81 filters as described above. The residual ^{125}I-alpha-BTX binding ability was determined as the ratio of the radioactivity bound in the presence and absence of the unlabeled ligand.

Under these conditions, the two competitive antagonists, gallamine and d-tubocurarine, efficiently inhibited ^{125}I-alpha-BTX binding with Ki of 7.4 and 7.5 mM, respectively, while neither agonist (carbamylcholine or nicotine) had any effect (Fig. 3). The lack of effect of the agonists or of different concentrations of NaCl shows that the inhibition was not caused by high ionic strength.

FPLC analysis.

The solubility and size of the recombinant protein were studied by FPLC on a Superose-12 column (Pharmacia – Biotech) using PBS buffer, pH 7.5, at a flow rate of 0.5 ml/min, followed by assay of the fractions for ^{125}I-alpha-BTX binding. As shown in Fig. 4, the protein migrated as a monomer with a molecular weight consistent with that estimated by SDS-PAGE.

mAb binding to Alpha1-210.

The binding to Alpha1-210 of several anti-MIR mAbs derived from rats immunized with intact AChR from either human muscle (mAb 190, 192, 185, 198, and 202) or Electrophorus electricus electric organ (mAb35) was tested by RIA. This study would be expected to provide useful information about the protein conformation, since some of the mAbs are known to bind exclusively to the native non-denatured AChR (mAb192, 190, and 35), while the binding of others is only partially conformation-dependent (mAb198, 195, and 202). In addition, we tested the binding of mAb64, an anti-alpha mAb not directed against the MIR. mAb25, which does not bind to mammalian AChR was used as a negative control.

Alpha1-210 (50 ng) was labeled by incubation for 3 h at 4°C with ^{125}I-alpha-BTX (50,000 cpm) in a total volume of 40 µl, then 10 µl of diluted mAb (1:200) containing 0.1 µl of normal rat serum as carrier was added and the samples incubated at 4°C for 15-18 h. Immune complexes were then precipitated by addition of 10 µl of rabbit anti-rat immunoglobulin serum and incubation for 1.5 h at 4°C, followed by centrifugation (4,000 rpm at 4°C for 10 min). The precipitates were washed three times with PBS-0.5% Triton-X 100 and the precipitated radioactivity counted.

The results of these RIA experiments are shown in Fig. 5A where, with the exception of the negative control (mAb25), all the mAbs tested (dilution 1:200) bound to the recombinant protein in RIA experiments. Overall, the facts that all tested mAbs were able to bind to recombinant Alpha1-210 suggests that the molecule assumes the correct conformation.

Binding of MG patients' autoantibodies to Alpha1-210.

As the antibody- and ligand-binding experiments indicated that the recombinant protein assumed the correct conformation, we were interested in studying the binding of anti-AChR autoantibodies from MG sera to Alpha1-210, since these antibodies are, in general, highly conformation-dependent. We screened 70 human sera: 50 MG and 20 healthy controls (5µl of human sera/sample). In order to determine the anti-alpha subunit specificities of the MG antisera, we analyzed, in parallel, their capacity to bind to recombinant Alpha1-210 (0.4pmole labeled with 150,000cpm ^{125}I-alpha-BTX) and to a solubilized hybrid AChR, HαTβγδ containing human alpha and *Torpedo* beta, gamma and delta subunits (17fmole labeled with 50,000cpm ^{125}I-alpha-BTX); since MG sera essentially do not bind to *Torpedo* AChR, their binding to the hybrid molecule represents binding to the alpha subunit in the hybrid AChR molecule, and we were therefore able to compare antibody binding to the alpha subunit inserted in the intact AChR molecule and to the isolated recombinant alpha subunit fragment. Thirty-six of the 50 MG sera (72%) were found to have high titers of anti-alpha autoantibodies (i.e. they immunoprecipitated an amount of radiolabeled hybrid AChR molecule greater than the mean of the controls + 3 standard deviations of that immunoprecipitated by healthy control sera) and 24 of these 36 (66%) recognized Alpha1-210 (i.e. they immunoprecipitated an amount of radiolabeled recombinant molecule greater than the mean of the controls + 3 standard deviations of that immunoprecipitated by healthy control sera). Interestingly, there was a strong linear correlation between the binding of human sera to ^{125}I-alpha-BTX-labeled Alpha1-210 and to the hybrid HαTβγδ AChR (r=0.74) (Fig. 6), suggesting that a major fraction of the anti-alpha autoantibodies in MG patients bind to recombinant Alpha1-210.

Example 2. Expression of the extracellular domains of beta, gamma, delta and epsilon subunits and their further study and use.

The N-terminal extracellular domain of the human muscle nicotinic AChR beta (Beta 1-222) and delta subunit (Delta 1-224) was enzymatically amplified by RT-PCR using total RNA extracted from TE671 cells using the primers: 5'-CCAGGCGTCGAATTCTCGGAG-3' (upstream for beta subunit), 5'-CAGGGTAGAATTCAGGCTTGCG-3' (downstream for beta subunit), 5'-GTGTGGCAGCGAATTCCTGAACGAG-3' (upstream for delta subunit) and 5'-GATGTAGAATTCTCACTTGCGGCGG-3' (downstream for delta subunit). The primers were constructed to contain EcoRI restriction sites (underlined). Using the appropriate restriction endonucleases, the purified cDNA fragment was subcloned into the expression vector pPIC9, so that the recombinant fragment was led by a signal peptide α-factor under the transcriptional control of the AOX promoter, which is induced by methanol, while at the C terminal end there was no sequence encoding the c-myc epitope or the polyhistidine (6xHis) tag. The resulting construct was linearized using StuI and transformed into the *Pichia pastoris* host strain GS115 or into the *Pichia pastoris* clone that expressed Alpha1-210 (example 1) in order to express Beta 1-222 and Delta1-224 alone or together with Alpha1-210 respectively. The transformed cells were plated on YPDS plus ampicillin (50µg/ml) for expression of Beta 1-222 and Delta1-224, or on YPDS plates plus ampicillin (50µg/ml) and zeocin (100 µg/ml) for expression of Beta 1-222 and Delta1-224 together with Alpha1-210. Single colonies of the transformants were initially inoculated into 4 ml of BMGY as described in example 1. After induction for 3 days with daily addition of methanol (0.5% v/v), as described above, the culture supernatants were tested for expression of Beta 1-222 and Delta1-224.

The vector pPIC9 (Invitrogen San Diego, CA) was modified by introducing the FLAG amino acid sequence (DYKDDDDK) immediately after the cleavage site of the secretion signal and has been shown to increase solubility of the expressed polypeptide in *Pichia*, probably due to the highly hydrophilic nature of the amino acids. This was performed by cloning the oligo 5'-GTAGATTACAAGGATGACGATGACAAAG-3' between the unique *SnaB*I and *Eco*RI sites of the vector. This allowed the subsequent cloning of any PCR product with an *Eco*RI site. The resulting plasmid was named pPIC9/FLAG.

The N-terminal extracellular domains of the human muscle nicotinic AChR gamma and epsilon subunits were also expressed in *Pichia pastoris* using the pPIC9 and pPIC9/FLAG vectors. PCR was performed on templates pcDNA3-gamma and pcDNA3-epsilon respectively (Dr. David Beeson, University of Oxford). Primers used were as follows. For gamma subunit, upstream primer 5'-GGTGTAGAATTCCGGAACCAGGAGGAGCGC-3' was used throughout with primers (a) 5'-ATAGTTTAGCGGCCGCTTACTTGC-GCTGGATGAGCAGG-3' for product gamma1-218, (b) 5'-ATAGTTTAGCGGCCGCTTAGTGATGGTGATGGTGATGCTTGCGCTGG ATGAGCAGG-3' for product gamma1-218His, (c)

5'-ATAGTTTAGCGGCCGCTTAGGGCTTGCGCTGGATGAGCAGG-3' for product gamma1-219, (d) 5'-ATAGTTTAGCGGCCGC-TTAGTGATGGTGATGGTGATGGGGCTTGCGCTGGATGAGCAGG-3' for product gamma1-219His. For epsilon subunit, upstream primer 5'-GGTGTAGAATTCAAGAACGAGGACTGCG-3' was combined with primers (a) 5'- ATAGTTTAGCGGCCGCTTACTTCCGGCG-GATGATGAGCGAG-3' for product epsilon1-219, (b) 5'-ATAGTTTAGCGGCCGCTTAGTGATGGTGATGGTGATGCTTCCGG CGGA-TGATGAGCGAG-3' for product epsilon1-219His, (c) 5'-ATAGTTTAGCGGCCGCTTACGGCTTCCGGCGGATGATGAGCGA G-3' for product epsilon1-220 and (d) 5'-ATAGTTTAGCGGCCGCTTAGTGATGGTGATGGTGATGCGGCTTC CGGCGGATGATGAG-CGAG-3' for product epsilon 1-220His. The constructs were linearized using StuI and transformed into the *Pichia pastoris* host strain GS115. The transformed cells were plated on YPDS plus ampicillin (50µg/ml). Single colonies of the transformants were initially inoculated into 4 ml of BMGY as described in example 1. After induction for 3 days with daily addition of methanol (0.5% v/v), as described above, the culture supernatants were tested for expression.

It is possible the simultaneous expression of any protein cloned in the pPIC9 vector with alpha1-210 which is present on vector pPICZalphaA (Example 1).

Example 3. Immobilization of the recombinant proteins on insoluble material and their use for immunoadsorption of MG antibodies.

To immunoadsorb autoantibodies from MG patient sera, 0.25mg Alpha1-210 mixed with 1.25mg BSA was bound to 0.5gr CNBr-Sepharose according to manufacturer's protocol. In parallel, 1.5mg BSA was bound to the same amount of CNBr-Sepharose under the same conditions. 2.5µl MG patient sera were incubated with 0.2µg immobilized Alpha1-210 mixed with BSA or the same amount of BSA at 4°C for 2-3h. 0.9 µl of these treated MG sera were subsequently used in a typical RIA MG diagnostic test. The percentage of immunoadsorption was estimated as the ratio between the reduction in immunoprecipitated radiolabeled human AChR after incubation with Alpha1-210 and the immunoprecipitated radiolabeled human AChR without incubation with Alpha1-210 (incubation with just BSA). 64 MG positive sera were assayed for binding to human AChR after incubation with immobilized Alpha1-210 or BSA. As shown in Fig.7, in 13 of the 64 (20%) MG sera more than 60% of the anti-AChR antibodies were found to be eliminated, while in 19 of these 64 (30%) the immunoadsorption was between 20-60%. For more stable fixation of the polypeptide on the column and for avoidance of release of any possible fragments of it, that polypeptide-Sepharose complex

is treated by a crosslinking reagent, in the present example 0.1% M glutaraldehyde.

Example 4. Expression of extracellular domain of human AChR alpha subunit in human cell cultures by the Semliki Forest Virus system (SFV) expression system and its further study and use.

The replication deficient-Semliki Forest Virus expression system is based on two plasmid vectors containing Semliki Forest virus cDNA. The pSFV-1 vector contains the region required for the production of the nsp1-4 replicase complex. A strong viral promoter is located at the 3'-end of the *nsp4* gene followed by a polylinker for the introduction of foreign genes. The pSFV helper vector encodes the viral structural genes (Liljeström and Garoff, 1991). Plasmid pSFV-1 containing the gene of interest can be used alone or together with the helper plasmid. In the former case (recombinant pSFV plasmid used alone) one only gets expression of the cloned gene whereas in the latter, one gets production of a recombinant viral stock which can be subsequently used to infect a larger number of host cells.

The DNA fragment encoding the extracellular domain of the human alpha AChR (Alpha 1-210) was amplified by PCR using upstream primer 5'-GCGGCCGCATGAAGGTTCTGTGGGC-TGCGTTGCTGGTCACATTCCTGGCAGGATGCCAGGCCTCCGAAC ATGAGACCCG-3' and downstream primer 5'-CCGAGCCTCGAGT-CAATGATGATGATGATGATGGTCGACG-3', on template pTEP1-1. The primers provide the *Not*I and *Xho*I sites (underlined) for further manipulation of the DNA. Upstream primer also provides the leader sequence of the human apolipoprotein E (ApoE); this sequence encodes the recognizable tag on ApoE, which allows for extracellular secretion of the protein and therefore it might also allow secretion of our protein. Dowstream primer encodes six additional histidine residues prior to the termination signal; the extra histidines will serve as a purification tool by use of nickel-agarose affinity chromatography. The obtained PCR product was digested using enzymes *Not*I and *Xho*I, filled in with the Klenow fragment of DNA polymerase I and cloned into *Sma*I site of the SFV-1 vector DNA.

Prior to the actual experiment with the SFV vector, the PCR product was additionally cloned into a commercial eucaryotic expression vector (pcDNA3.0+, Invitrogen) and the recombinant plasmid was transfected into human HEK293 cells. Approximately $2x10^6$ transfected cells were allowed to express the protein of interest. Cells and medium supernatant were collected after two days. Cells were washed in cocktail buffer (1x PBS pH7.4 with PMSF 1mM, aprotinin 5units/ml, leupeptin 5micrograms/ml, iodoacetamide 1mM). The medium supernatant (12ml) was concentrated 5 times to a final volume of 2.5 ml and dialysed against PBS buffer at 4°C. To extract the receptor, the cell pellet was resuspended in 0.5ml of cocktail-2% Triton-X100 and the mixture was left at 4°C with mild agitation. The mixture was subsequently centrifuged at 15000g for 15 min and the

supernatant was tested for ^{125}I-alpha-bungarotoxin binding. The filter assay technique (as described in Example 1) was used to test whether the expressed protein was secreted in the medium supernatant or retained within the cell (in either the cytoplasm or the membrane). The results indicated that good expression of functional human extracellular domain of alpha subunit is achieved, but that the protein is present intracellularly.

Having established that the cloned product is functional, the recombinant SFV plasmid is linearized at *Spe*I site, purified and used in an *in vitro* mRNA synthesis using the mMESSAGE mMACHINE kit (Ambion Inc.) Approximately 20 micrograms of mRNA produced is obtained and used to electroporate 0.7 ml of 10^7 cells/ml HEK293 cells. We only use the recombinant SFV-1 vector without the helper plasmid at this stage. Both the cells and the supernatant are collected two days post-electroporation. Cells are washed, disrupted by use of the above described cocktail-2% Triton X100 buffer and further treated as described above. The medium supernatant is collected, concentrated and dialyzed as described above. The results of filter assay experiments using the concentrated/dialyzed medium supernatant and the supernatant of the extracted cells showed that the protein is expressed at satisfactory levels. The protein appears to be functional and displaying a good ability to bind 125I-alpha bungarotoxin.

CLAIMS

1. The procedure which consists of the production and purification of recombinant subunits or their fragments containing extracellular parts of human or animal acetylcholine receptor subunits, with capacity to bind large fractions of anti-AChR antibodies from MG patients.
2. The molecules of claim 1, which contain the extracellular N-terminal parts of human AChR subunits.
3. The procedure that consists of the immobilization of the molecules of claims 1 and 2 on insoluble carriers.
4. Use of the immobilized molecules of claim 3 for *in vitro* elimination of antibodies against the AChR from sera of myasthenic patients.
5. Use of the immobilized molecules of claim 3 for therapeutic *ex vivo* elimination/apheresis of anti-AChR antibodies from the blood of myasthenic patients.
6. The procedure which consists of the production and purification of alpha subunit of human acetylcholine receptor or its fragments in yeast *Pichia pastoris*, with capacity to bind large fractions of anti-AChR antibodies from MG patients.
7. The molecules of claim 6, which contain the extracellular N-terminal parts of human AChR alpha subunit.
8. The procedure that consists of the immobilization of the molecules of claims 6 and 7 on insoluble carriers.
9. Use of the immobilized molecules of claim 8 for *in vitro* elimination of antibodies against the AChR from sera of myasthenic patients.
10. Use of the immobilized molecules of claim 8 for therapeutic *ex vivo* elimination/apheresis of anti-AChR antibodies from the blood of myasthenic patients.
11. The procedure which consists of the production and purification of beta subunit of human acetylcholine receptor or its fragments in yeast *Pichia pastoris*, with capacity to bind large fractions of anti-AChR antibodies from MG patients.
12. The molecules of claim 11, which contain the extracellular N-terminal parts of human AChR beta subunit.
13. The procedure that consists of the immobilization of the molecules of claims 11 and 12 on insoluble carriers.
14. Use of the immobilized molecules of claim 13 for *in vitro* elimination of antibodies against the AChR from sera of myasthenic patients.
15. Use of the immobilized molecules of claim 13 for therapeutic *ex vivo* elimination/apheresis of anti-AChR antibodies from the blood of myasthenic patients.
16. The procedure which consists of the production and purification of gamma subunit of human acetylcholine receptor or its fragments in yeast *Pichia pastoris*, with capacity to bind large fractions of anti-AChR antibodies from MG patients.
17. The molecules of claim 16, which contain the extracellular N-terminal parts of human AChR gamma subunit.
18. The procedure that consists of the immobilization of the molecules of claims 16 and 17 on insoluble carriers.

19. Use of the immobilized molecules of claim 18 for *in vitro* elimination of antibodies against the AChR from sera of myasthenic patients.
20. Use of the immobilized molecules of claim 18 for therapeutic *ex vivo* elimination/apheresis of anti-AChR antibodies from the blood of myasthenic patients.
21. The procedure which consists of the production and purification of delta subunit of human acetylcholine receptor or its fragments in yeast *Pichia pastoris*, with capacity to bind large fractions of anti-AChR antibodies from MG patients.
22. The molecules of claim 21, which contain the extracellular N-terminal parts of human AChR delta subunit.
23. The procedure that consists of the immobilization of the molecules of claims 21 and 22 on insoluble carriers.
24. Use of the immobilized molecules of claim 23 for *in vitro* elimination of antibodies against the AChR from sera of myasthenic patients.
25. Use of the immobilized molecules of claim 23 for therapeutic *ex vivo* elimination/apheresis of anti-AChR antibodies from the blood of myasthenic patients.
26. The procedure which consists of the production and purification of epsilon subunit of human acetylcholine receptor or its fragments in yeast *Pichia pastoris*, with capacity to bind large fractions of anti-AChR antibodies from MG patients.
27. The molecules of claim 26, which contain the extracellular N-terminal parts of human AChR epsilon subunit.
28. The procedure that consists of the immobilization of the molecules of claims 26 and 27 on insoluble carriers.
29. Use of the immobilized molecules of claim 28 for *in vitro* elimination of antibodies against the AChR from sera of myasthenic patients.
30. Use of the immobilized molecules of claim 28 for therapeutic *ex vivo* elimination/apheresis of anti-AChR antibodies from the blood of myasthenic patients.

Title:

Production of recombinant fragments of muscle acetylcholine receptor and their use for *ex vivo* immunoadsorption of anti-ACh receptor antibodies from myasthenic patients

Abstract

A procedure which involves the production of extracellular domains (or larger parts including the said domains) of human acetylcholine receptor (AChR) expressed in heterologous or homologous systems and obtained in sufficiently folded state appropriate for binding of major fractions of anti-AChR antibodies in myasthenia gravis (MG) patients. This procedure allows the *ex vivo* selective elimination of the antibodies against human muscle acetylcholine receptor (AChR), as a replacement and much improvement of the non-specific plasmapheresis or Ig apheresis, for the temporary repeated treatment of the disease MG. These domains are immobilized on insoluble material and are used for the immunoadsorption of the said antibodies.

20020100190

FIGURES

Figure. 1

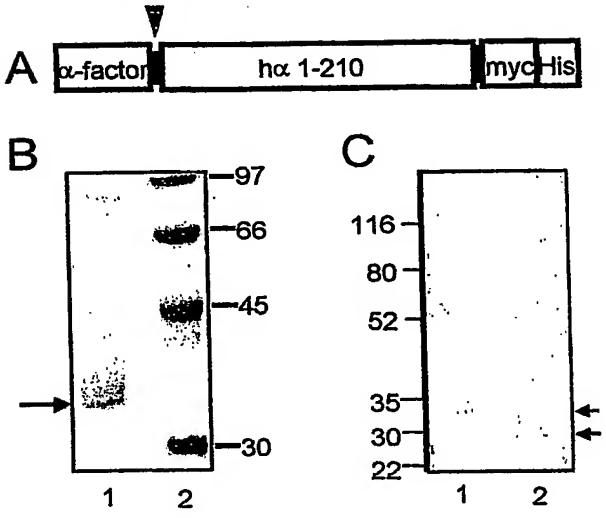


Figure. 2

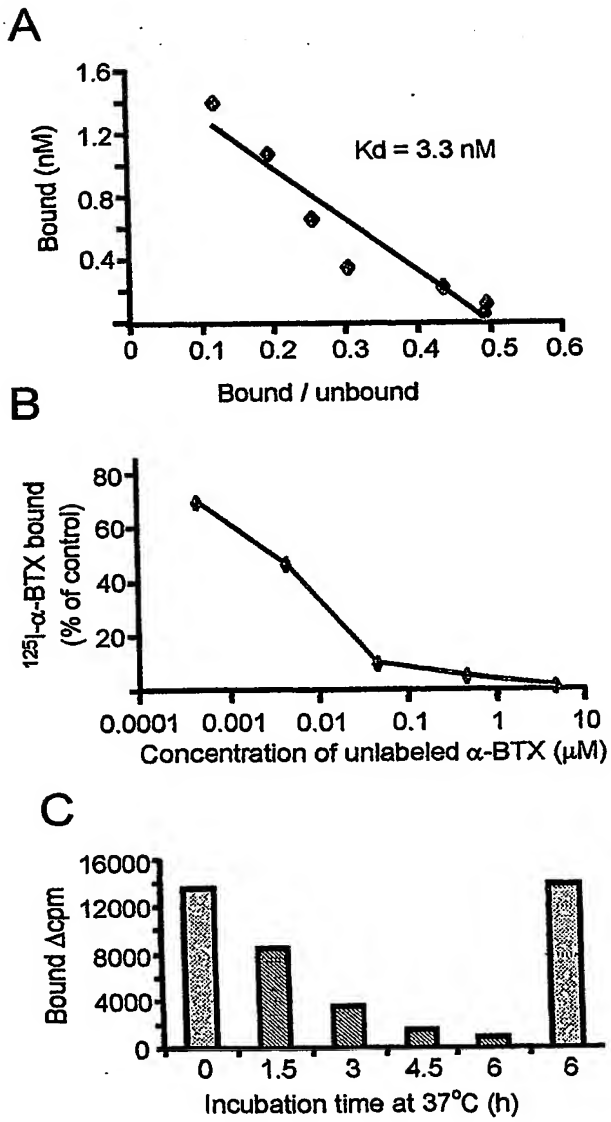

A
1.6
1.2
0.8
0.4
Bound (nM)
Kd = 3.3 nM
0
0.1
0.2
0.3
0.4
0.5
0.6
Bound / unbound
B
80
60
40
20
125I-α-BTX bound (% of control)
0.0001
0.001
0.01
0.1
1
10
Concentration of unlabeled α-BTX (μM)
C
16000
12000
8000
4000
0
Bound Δcpm
0
1.5
3
4.5
6
6
Incubation time at 37°C (h)

Figure. 3

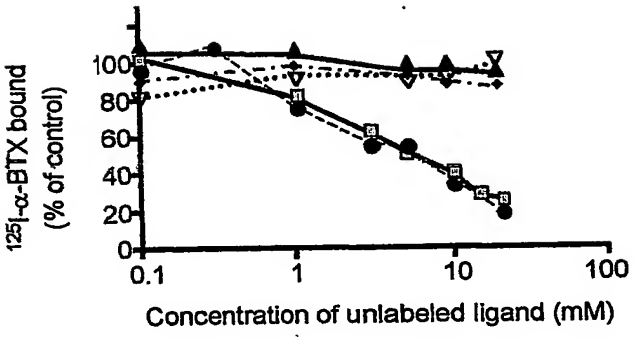

^{125}I-α-BTX bound
(% of control)
100
80
60
40
20
0
0.1
1
10
100
Concentration of unlabeled ligand (mM)

Figure. 4

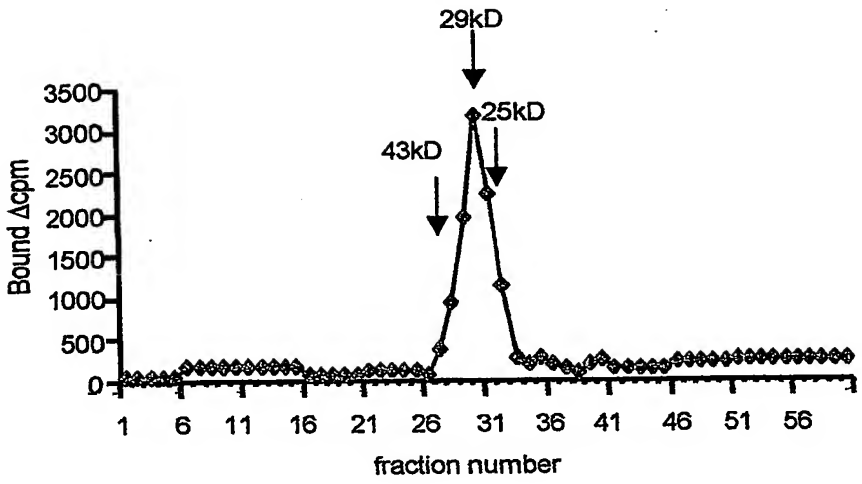

29kD
25kD
43kD
Bound Δcpm
3500
3000
2500
2000
1500
1000
500
0
1
6
11
16
21
26
31
36
41
46
51
56
fraction number

Figure. 5

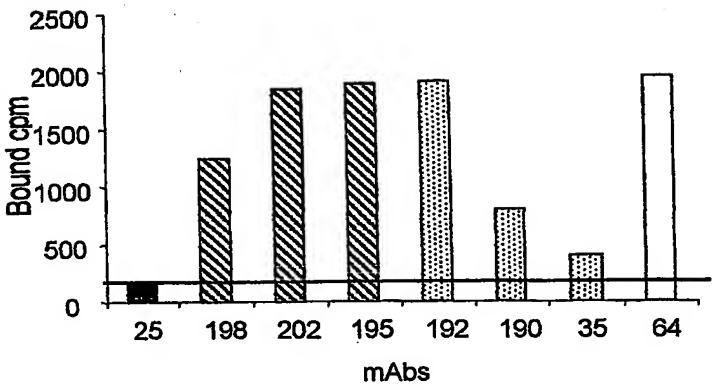

2500
2000
1500
1000
500
0
Bound cpm
25
198
202
195
192
190
35
64
mAbs

Figure. 6

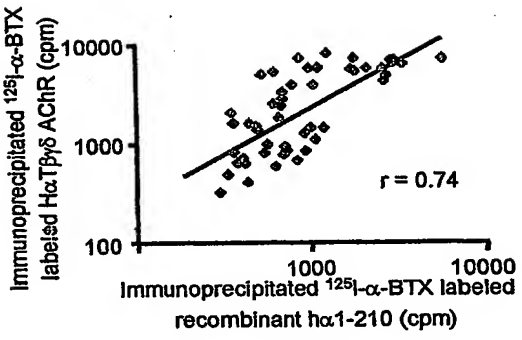
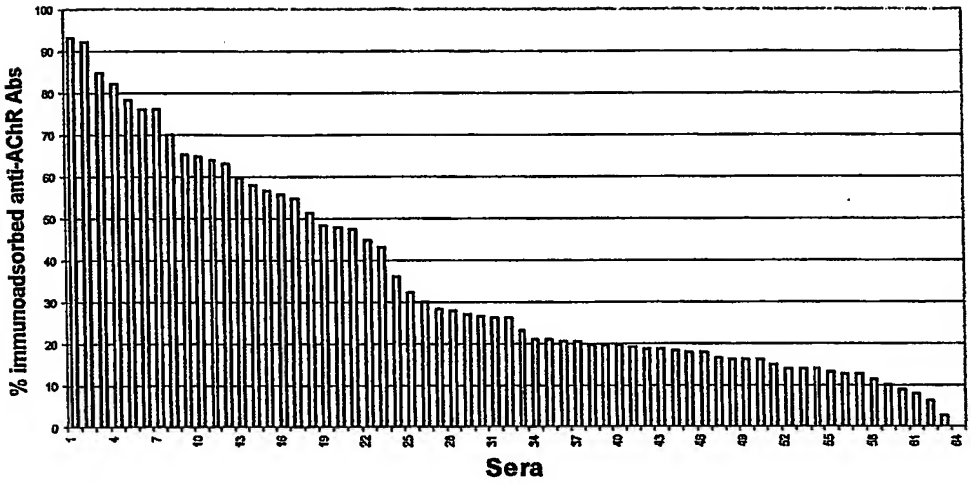

Immunoprecipitated ^{125}I-α-BTX labeled HαTβγδ AChR (cpm)
10000
1000
100
r = 0.74
1000
10000
Immunoprecipitated ^{125}I-α-BTX labeled recombinant hα1-210 (cpm)

Figure.7


% immunoadsorbed anti-AChR Abs
100
90
80
70
60
50
40
30
20
10
0
1
4
7
10
13
16
19
22
25
28
31
34
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